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(54) Title: CHIMERIC PROTEINS WITH CELL-TARGETING SPECIFICITY AND APOPTOSIS-DINDUCING ACTIVITIES

(57) Abstract

The present invention relates to chimeric proteins with cell-targeting specificity and apoptosis-inducing activities. In particular, the invention is illustrated by a recombinant chimeric protein between human interleukin-2 (IL2) and Bax. The chimeric protein specifically targets IL2 receptor (IL2R)-expressing cells and induces cell-specific apoptosis.

CHIMERIC PROTEINS WITH CELL-TARGETING SPECIFICITY AND APOPTOSIS-INDUCING ACTIVITIES

1.   INTRODUCTION

5   The present invention relates to chimeric proteins with
cell-targeting specificity and apoptosis-inducing activities.
In particular, the invention is illustrated by a recombinant
chimeric protein between human interleukin-2 (IL2) and Bax.
The chimeric protein specifically targets IL2 receptor
10 (IL2R)-expressing cells and induces cell-specific apoptosis.
In accordance with the invention, chimeric proteins may be
generated between any molecule that binds a specific cell
type and an apoptosis-inducing protein. Such chimeric
proteins are useful for selectively eliminating specific cell
15 types in vitro and in vivo, and may be used in the treatment
of autoimmunity, cancer and infectious diseases such as viral
infections.

2.   BACKGROUND OF THE INVENTION

20   ### 2.1. IMMUNOTOXINS
The advent of the monoclonal antibody technology and
recombinant DNA technology have led to the discovery of
numerous cell surface molecules associated with specific cell
populations. Based on the expression pattern of these
25 molecules, recombinant immunotoxins have been constructed to
specifically target and destroy the cells that express such
molecules. Recombinant immunotoxins are a class of targeted
molecules designed to recognize and specifically destroy
cells expressing specific receptors, such as cancer cells and
30 cells involved in many disorders of the immune system.
Generally, immunotoxins utilize a bacterial or plant toxin to
destroy the unwanted cells. These molecules are designed and
constructed by gene fusion techniques and are composed of
both the cell targeting and cell killing moieties, a
35 combination that makes these agents potent molecules for
treatment. Examples of immunotoxins are growth factors or
antigen-binding domains of antibody, including the Fv portion

of an antibody (single-chain immunotoxins) fused to various
mutant forms of toxin molecules. However, over the years it
has become clear that treatment with such "magic bullets" for
targeted immunotherapy possesses still many problems and new

5 approaches are needed to produce improved recombinant
immunotoxins.

Each recombinant immunotoxin displays some nonspecific
toxicity and at sufficiently high concentrations damages
normal cells that do not express the specific target antigen.

10 This non-specific toxicity of immunotoxins is the dose-
limiting factor in immunotoxin therapy. Which tissues are
affected by nonspecific toxicity is dependent on the
particular toxin used for immunotoxin preparation, and the
ability of immunotoxins to penetrate into tissues and tumors

15 is largely dependent on the size of the immunotoxins.

Large stable conjugated immunotoxins persist for long
periods in blood vessels ($T_{\frac{1}{2}}5$-15 hour), thus endothelial cells
are exposed to high toxin concentrations which may lead to
endothelial cell damage. Smaller molecules, such as

20 recombinant immunotoxins which rapidly leave the vascular
system, would presumably have different toxicity. In humans,
immunotoxins made with ricin and other ribotoxins, as well as
with Pseudomonas exotoxin A (PE), Diphtheria toxin (DT) and
their truncated derivatives have produced a variety of

25 toxicities. These include vascular leak syndrome (mainly
ricin immunotoxins) as well as liver toxicity (PE-derived
immunotoxins). Vascular leak syndrome observed with ricin
immunotoxins in animals and man may be explained by specific
binding of ricin A-chain to endothelial cells and subsequent

30 killing of the cells and damage to the vessels. The
nonspecific liver-toxicity of PE immunotoxins is likely to be
due to easy access and very rapid nonspecific uptake and
internalization of proteins by hepatocytes. However, it is
also possible that PE contains, in addition to the specific

35 cell-binding site (Domain I) which is removed in most
immunotoxins, an additional site which could be recognized

with low affinity by hepatocytes, thus accounting for liver
toxicity.

        Another major impediment with immunotoxins in their
clinical application is the human immune response against
5 them, mainly toward the toxin moiety. Bacterial toxins like
PE and DT are highly immunogenic and cannot be humanized with
standard techniques. Usage of DT-derived immunotoxins is
limited because most people in developed countries have been
vaccinated against DT and many adults have neutralizing
10 antibodies to DT. Immunogenicity is a problem to which so
far no practical solution has been found. Reduced
immunogenicity of these molecules would greatly improve the
clinical application of immunotoxins.

        An example of the successful use of an immunotoxin is
15 the elimination of activated T cells which express high
affinity IL2 receptors (IL2R), whereas normal resting T cells
and their precursors do not. An immunotoxin made of IL2
could theoretically eliminate IL2R-expressing leukemia cells
or IL2R-expressing immune cells involved in various disease
20 states while not destroying IL2R negative normal cells,
thereby preserving the full repertoire of antigen receptors
required for T cell immune responses.

        A chimeric protein, IL2-PE40, was produced and shown to
eliminate activated T cells (Lorberboum-Galski et al., 1988,
25 Proc. Natl. Acad. Sci. U.S.A. 85:1922). IL2-PE40 was
extremely cytotoxic to IL2R-expressing cell lines of human,
ape and murine origin. It was also extremely cytotoxic to
Con A-stimulated mouse and rat spleen cells, and had a
suppressive effect against antigen-activated mouse cells and
30 the generation of cytotoxic T cells in mixed lymphocyte
cultures (Lorberboum-Galski et al., 1988, J. Biol. Chem.
263:18650-18656; Ogata et al., 1988, J. Immunol. 41:4224-
4228; Lorberboum-Galski et al., 1990, J. Bio. Chem.
265:16311-16317).

35   A highly purified IL2-PE40 preparation (Bailon et al.,
1988, Biotechnol. 6:1326-1329) was shown to (a) delay and
mitigate adjuvant induced arthritis in rats (Case et al.,

1989, Proc. Natl. Acad. Sci. U.S.A. 86:287-291), (b)
significantly prolong the survival of vascularized heart
allograft in mice (Lorberboum-Galski et al., 1989, Proc.
Natl. Acad. Sci. U.S.A. 86:1008-1012) and corneal allografts
5 in rats (Herbort et al., 1991, Transplant. 52:470-474), (c)
reduce the incidence and severity of experimental autoimmune
uveoretinitis in rats (Roberge et al., 1989, J. Immunol.
143:3498-3502), (d) suppress the growth of an IL2R bearing T
cell lymphoma in mice (Kozak et al., 1990, J. Immunol.
10 145:2766-2771) and (e) prevent the development of
experimental allergic encephalomyelitis, a T cell mediated
disease of the central nervous system, in rats and mice
(Beraud et al., 1991, Cell. Immunol. 133:379-389; Rose et
al., 1991, J. Neuroimmunol. 32:209-217). However, such
15 immunotoxin still suffers from the same deficiencies outlined
above, particularly non-specific toxicity and immunogenicity
in the human host.

2.2. APOPTOSIS-INDUCING PROTEINS

20   The development of multilineage organisms and the
maintenance of homeostasis within tissues both require
tightly regulated cell death. The ability of an individual
cell to execute a suicidal response following a death
stimulus varies markedly during its differentiation. Both
25 positive and negative regulators of programmed cell death
(apoptosis) have been identified.

A high percentage of follicular lymphomas have a
characteristic chromosomal translocation, which places the
proto-oncogene, Bcl-2 next to the immunoglobulin heavy chain
30 locus, resulting in deregulation of Bcl-2 expression. Bcl-2
was found to function as a repressor of programmed cell death
(Vaux et al., 1988, Nature 334:440-442). Recently, other
Bcl-2 homologues were shown to inhibit apoptosis. However,
one such homologue, Bax, mediates an opposite effect by
35 accelerating apoptosis. An expanding family of Bcl-2 related
proteins has recently been noted to share homology that is
principally, but not exclusively, clustered within two

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conserved regions known as *Bcl-2* homology domains 1 and 2
(*BH1* and *BH2*) (Oltvai et al., 1993, Cell 74:609-619; Boise et
al., 1993, Cell 74:597-608; Kozopas et al., 1993, Proc. Natl.
Acad. Sci. U.S.A. 90:3516-3520; Lin et al., 1993, J. Immunol.
5 151:1979-1988). Members of the *Bcl* family include *Bax*, *Bcl-*
X$_L$, *Mcl-1*, *A1* and several open reading frames in DNA viruses.
Another conserved domain in Bax, distinct from BH1 and BH2
was identified and termed BH3. This domain mediates cell
death and protein binding functions (Chittenden et al., 1995,
10 EMBO J. 14:5589-5596). Another member of the pro-apoptotic
proteins contains only the BH3 domain, implying that this
particular domain may be uniquely important in the promotion
of apoptosis (Diaz et al., 1997, J. Biol. Chem. 272:11350-
11355).

15   Bax homodimerizes and forms heterodimers with BCL-2 *in*
vivo. Overexpressed Bax overcomes the death repressor
activity of Bcl-2 (Oltvai et al., 1993, Cell 74:609-619). It
was found that levels of Bax expression higher than Bcl-2 in
bladder tumors was correlated with a better outcome for
20 patients. Early relapses were much more frequently observed
in patients whose tumors expressed more *Bcl-2* than *Bax* mRNA
(Gazzaniga et al., 1996, Int. J. Cancer 69:100-104).

      Recently it was reported that Bax-alpha, a splice
variant of Bax was expressed in high amount in normal breast
25 epithelium, whereas only weak or no expression could be
detected in 39 out of 40 cancer tissue samples examined
(Bargou et al., 1996, J. Clin. Invest. 97:2651-2659). Of
interest, downregulation of Bax-alpha was found in different
histological subtypes. Furthermore, when Bax-alpha was
30 transfected into breast cancer cell lines under the control
of a tetracycline-dependent expression system, Bax restored
sensitivity of the cancer cells toward both serum starvation
and APO-I/Fas-triggered apoptosis, and significantly reduced
tumor growth in SCID mice. Therefore, it was proposed that
35 dysregulation of apoptosis might contribute to the
pathogenesis of breast cancer at least in part due to an

imbalance between members of the *Bcl-2* gene family (Bargou et al., 1996, J. Clin. Invest. 97:2651-2659).

In another study, the expression of *Bax* was investigated in 52 cases of Hodgkin's disease in parallel with Epstein-
5 Barr virus, and was compared with the immunodetection of other apoptosis-regulating proteins, Mcl-1, Bcl-2 and Bcl-x. *Bax* expression was frequently detected in Hodgkin's disease, providing an explanation for the good chemoresponses generally obtained for patients with this neoplastic disorder
10 (Rigal-Haguet et al., 1996, Blood 87:2470-2475).

Additional members of this growing family of apoptosis inducing proteins have been cloned and identified. Bak is a new member of the Bcl-2 family which is expressed in a wide variety of cell types and binds to the Bcl-2 homologue Bcl-x2
15 in yeast (Farrow et al., 1995, Nature 374:731-733; Chittenden et al., 1995, Nature 374:733). A domain in Bak was identified as both necessary and sufficient for cytotoxicity activity and binding to Bcl-xl. Sequences similar to this domain that are distinct from BH1 and BH2 have been
20 identified in Bax and Bipl. This domain was found to be of central importance in mediating the function of multiple cell death-regulatory proteins that interact with Bcl-2 family members (Chittenden et al., 1995, EMBO J. 14:5589-5596).

Overexpression of Bak in sympathetic neurons deprived of
25 nerve growth factor accelerated apoptosis and blocked the protective effect of co-injected E1B 19K. The adenovirus E1B 19K protein is known to inhibit apoptosis induced by E1A, tumor-necrosis factor-alpha, FAS antigen and nerve growth factor deprivation (Farrow et al., 1995, Nature 374:731-733).
30 Expression of Bak induced rapid and extensive apoptosis of serum-deprived fibroblasts, thus raising the possibility that Bak is directly involved in activating the cell death machinery (Chittenden et al., 1995, Nature 374:733-736). It was also reported that in the normal and neoplastic colon,
35 mucosal expression of immunoreactive Bak co-localized with sites of epithelial cell apoptosis. Induction of apoptosis in the human colon cancer cell line HT29 and the rat normal

small intestinal cell line 1EC 18 in culture was accompanied
by increased Bak expression without consistent changes in
expression of other Bcl-2 homologous proteins (Moss et al.,
1996, Biochem. Biophys. Res. Commun. 223:199-203).

5   Therefore, Bak was also suggested to be the endogenous Bcl-2
family member best correlated with intestinal cell apoptosis
(Moss et al., 1996, Biochem. Biophys. Res. Commun. 223:119-
203).

         Unlike Bax, however, Bak can inhibit cell death in an
10 Epstein-Barr-virus-transformed cell line.   Tissues with
unique distribution of Bak messenger RNA include those
containing long-lived, terminally differentiated cell types
(Krajewski et al., 1996, Cancer Res. 56:2849-2855),
suggesting that cell-death-inducing activity is broadly
15 distributed, and that tissue-specific modulation of apoptosis
is controlled primarily by regulation of molecules that
inhibit apoptosis (Kiefer et al., 1995, Nature 374:736-739).

         Another member of the Bcl2 family is Bad that possesses
the key amino acid motifs of BH1 and BH2 domains.   Bad lacks
20 the classical C-terminal signal-anchor sequence responsible
for the integral membrane positions of other family members.
Bad selectively dimerizes with $Bcl-x_L$ as well as Bcl-2, but
not with Bax, Bcl-Xs-Mcl1, A1 or itself.   Bad reverses the
death repressor activity of $Bcl-X_L$, but not that of Bcl-2
25 (Yang et al., 1995, Cell 80:285-291; Ottilie et al., 1997, J.
Biol. Chem. 272:30866-30872; Zha et al., 1997, J. Biol. Chem.
272:24101-24104).

         Another member is Bik which interacts with the cellular
survival-promoting proteins, Bcl-2 and $Bcl-X_L$ as well as the
30 viral survival-promoting proteins, Epstein Barr virus-BHRF1
and adenovirus E1B-19kDa.   In transient transfection assays,
Bik promotes cell death in a manner similar to other death-
promoting members of the Bcl-2 family, Bax and Bak.   This
death-promoting activity of Bik can be suppressed by
35 coexpression of Bcl-2, $Bcl-X_L$, EBV-BHRF1 and E1B-19 kDa
proteins suggesting that Bik may be a common target for both
cellular and viral anti-apoptotic proteins.   While Bik does

not contain overt homology to the BH1 and BH2 conserved
domains characteristic of the Bcl-2 family, it shares a 9
amino acid domain (BH3) with Bax and Bak which may be a
critical determinant for the death-promoting activity of
5 these proteins (Boyd et al., 1995, Oncogene 11:1921-1928; Han
et al., 1996, Mol. Cell. Biol. 16:5857-5864).

The Bcl-2 family is composed of various pairs of
antagonist and agonist proteins that regulate apoptosis.
Whether their function is interdependent is uncertain. Using
10 a genetic approach to address this question, Knudson et al.
(1997, Nature Genetics 16:358-363), recently utilized gain-
and loss of- function models of Bcl-2 and Bax, and found that
apoptosis and thymic hypoplasia, characteristic of Bcl-2-
deficient mice, are largely absent in mice also deficient in
15 Bax. A single copy of Bax promoted apoptosis in the absence
of Bcl-2. In contrast, overexpression Bcl-2 still repressed
apoptosis in the absence of Bax. While an in vivo
competition exists between Bax and Bcl-2, each is able to
regulate apoptosis independently. Bax has been shown to form
20 channels in lipid membranes and trigger the release of
liposome-encapsulated carboxyluorescein at both neutral and
acidic pH. At physiological pH, release could be blocked by
Bcl-2. In planer lipid bilayers, Bax formed pH- and voltage-
dependent ion-conduction channels. Thus, the pro-apoptotic
25 effects of Bax may be elicited through an intrinsic pore-
forming activity that can be antagonized by Bcl-2 (Antonsson
et al., 1997, Science 277:370-372). Two other members of
this family, Bcl-2 and Bcl-1, were also shown to form pores
in lipid membranes (Schendel et al., 1997, Proc. Natl. Acad.
30 Sci. U.S.A. 94:5113-5118).

Prior to the present invention, a fusion protein
containing a Bcl-2 pro-apoptotic member was not reported, nor
was it predictable if such a molecule could retain biological
activites when added to a cell exogenously to induce
35 apoptosis.

3.   **SUMMARY OF THE INVENTION**

The present invention relates to chimeric proteins with cell-targeting specificity and apoptosis-inducing activities. The chimeric proteins of the invention are composed of a

5 cell-specific targeting moiety and an apoptosis-inducing moiety. The cell-specific targeting moiety provides cell-specific binding properties to the chimeric protein, while the apoptosis-inducing moiety induces programmed cell death upon entry into a target cell. It is preferred that the

10 chimeric proteins of the invention be produced by recombinant expression of a fusion polynucleotide between a coding sequence of a cell-targeting moiety and a coding sequence of an apoptosis-inducing protein. Such chimeric proteins are likely to be superior to the immunotoxins currently used in

15 the art because they are of human origin and thus are expected to have reduced immunogenicity in a human recipient. In addition, chimeric proteins kill target cells by inducing apoptosis which does not cause a release of cellular organelles into the extracellular environment to result in an

20 inflammatory response. When cells die by the apoptotic pathway, they shrink and condense, but the organelles and plasma membranes retain their integrity, and the dead cells are rapidly phagocytosed by neighboring cells or macrophages before there is leakage of the cells' contents, thereby

25 eliciting minimal tissue or systemic response.

The invention also relates to pharmaceutical compositions of the chimeric proteins, methods of producing such proteins, and methods of using the same *in vitro* and *in vivo*, especially for eliminating specific undesirable target

30 cells, and for the treatment of a variety of disease conditions as well as the use of the proteins for disease diagnosis.

The invention is based, in part, on the Applicants' discovery that a partially purified recombinant chimeric

35 protein, IL2-Bax, specifically targets IL2R$^+$ cells, which include but are not limited to, T cells, B cells, monocytes and natural killer cells. The protein kills target cells by

inducing apoptosis of these cells. A wide variety of uses
are encompassed by the present invention, including but not
limited to, the treatment of autoimmunity, transplantation
rejection, graft-versus-host disease, cancer,
5 hypersensitivity, and infectious diseases.

4. __BRIEF DESCRIPTION OF THE DRAWINGS__

Figure 1:   Construction of the pSY1 plasmid that
                 encodes a chimeric protein composed of
10   IL2 and Bax-α (designated IL2-Bax) under
                 the control of the T7 promoter. The
                 numbers represent the corresponding amino
                 acids.

Figure 2:   Nucleotide sequence (SEQ ID NO:1) of a
15   coding sequence for chimeric protein,
                 IL2-Bax, and its deduced amino acid
                 sequence (SEQ ID NO:2).

Figure 3:   SDS-PAGE analysis of cell fractions
                 containing the IL2-Bax chimeric protein.
20   IL2-Bax was overexpressed in *E. coli* BL21
                 (λDE3) and subfractionated as described
                 in Section 6.1.2., *infra*. Samples of
                 each subfraction were mixed with Laemmli
                 sample buffer and loaded on a 10%
25   polyacrylamide gel.
                 Lanes: 1, insoluble fraction treated
                 with extraction buffer B containing SDS.
                 2, insoluble fraction treated with
                 extraction buffer C containing urea. 3,
30   insoluble fraction treated with
                 extraction buffer A, containing Gu-HCL.
                 4, soluble fraction. M, markers. Arrow
                 indicates the position of the IL2-Bax
                 chimeric protein.

35 Figure 4:   Immunobloting of fractions containing
                 IL2-Bax with antibodies to the Bax
                 protein.

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Lanes: 1, soluble fraction.   2,
insoluble fraction treated with
extraction buffer A containing Gu-HCL.
3, insoluble fraction treated with
extraction buffer C containing urea.   4,
insoluble fraction treated with
extraction buffer B containing SDS.   5, A
protein extract of MCF-7 cells known to
express the Bax protein (indicated by the
*).   Arrow indicates the position of the
IL2-Bax chimeric protein.

Figures 5A&B: Effect of IL2-Bax on protein synthesis in
target (5A) and non-target (5B) cell
lines.   IL2-Bax (insoluble fraction
treated with Gu-HCL) was added at
different concentrations to the various
cell lines.   [³H] leucine incorporation
into cellular protein was measured.
Results are expressed as percent of
control cells not exposed to IL2-Bax.

Figure 6A-D: FACS analysis of fresh lymphocytes
exposed to IL2-Bax.   Fresh lymphocytes
were separated, exposed to the IL2-Bax
chimeric protein and apoptotic cells were
analyzed by FACS.   The cells were
untreated (6A) or treated with
dexamethasone (6B), IL2-Bax at 1 μg/ml
(6C) or IL2-Bax at 10 μg/ml (6D).

Figure 7A-C: FACS analysis of fresh lymphocytes
exposed to IL2-Bax.   Fresh lymphocytes
were separated, exposed to the IL2-Bax
chimeric protein and apoptotic cells were
analyzed by FACS.   The cells were
untreated (7A) or treated with IL2-Bax at
1 μg/ml (7B) or IL2-Bax at 10 μg/ml (7C).

Figure 8A-E: FACS analysis of HUT102 exposed to IL2-
Bax.   HUT102 cells were exposed to IL2-

Bax chimeric protein and analyzed by FACS
to characterize apoptotic cells. Results
are expressed in a logarithmic mode. The
cells were untreated (8A) or treated with

5   IL2-Bax at 1 μg/ml (8B), IL2-Bax at 2
μg/ml (8C), IL2-Bax at 4 μg/ml (8D) or
IL2-Bax at 5 μg/ml (8E).

Figure 9A-C: FACS analysis of CEM cells exposed to
IL2-Bax. CEM cells were exposed to IL2-

10   Bax chimeric protein and analyzed by FACS
to characterize apoptotic cells. Results
are expressed in a logarithmic mode. The
cells were untreated (9A) or treated with
IL2-Bax at 1 μg/ml (9B) or IL2-Bax at 5

15   μg/ml (9C).

5.   DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to chimeric proteins,
pharmaceutical compositions of chimeric proteins, methods of
20 producing a chimeric protein and methods of using the
protein. For clarity of discussion, the specific
compositions, procedures and methods described herein are
exemplified using IL2 and Bax; they are merely illustrative
for the practice of the invention. Analogous procedures and
25 techniques are equally applicable to constructing other
chimeric proteins between any cell-specific targeting moiety
and apoptosis-inducing moiety.

5.1. CONSTRUCTION OF CHIMERIC MOLECULES

30   While the chimeric proteins of the present invention may
be produced by chemical synthetic methods or by chemical
linkage between the two moieties, it is preferred that they
are produced by fusion of a coding sequence of a cell-
specific targeting moiety and a coding sequence of an
35 apoptosis-inducing protein under the control of a regulatory
sequence which directs the expression of the fusion
polynucleotide in an appropriate host cell. The fusion of

two full length coding sequences can be achieved by methods
well known in the art of molecular biology. It is preferred
that a fusion polynucleotide contain only the AUG translation
initiation codon at the 5' end of the first coding sequence
5 without the initiation codon of the second coding sequence to
avoid the production of two encoded product. In addition, a
leader sequence may be placed at the 5' end of the
polynucleotide in order to target the expressed product to a
specific site or compartment within a host cell to facilitate
10 secretion or subsequent purification after gene expression.
The two coding sequences can be fused directly without any
linker or by using a flexible polylinker composed of the
pentamer Gly-Gly-Gly-Gly-Ser repeated 1 to 3 times. Such
linker has been used in constructing single chain antibodies
15 (scFv) by being inserted between V_H and V_L (Bird et al., 1988,
Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad.
Sci. U.S.A. 85:5979-5883). The linker is designed to enable
the correct interaction between two beta-sheets forming the
variable region of the single chain antibody. Other linkers
20 which may be used include Glu-Gly-Lys-Ser-Ser-Gly-Ser-Gly-
Ser-Glu-Ser-Lys-Val-Asp (Chaudhary et al., 1990, Proc. Natl.
Acad. Sci. U.S.A. 87:1066-1070) and Lys-Glu-Ser-Gly-Ser-Val-
Ser-Ser-Glu-Gln-Leu-Ala-Gln-Phe-Arg-Ser-Leu-Asp (Bird et al.,
1988, Science 242:423-426).
25

5.1.1.   CELL-SPECIFIC TARGETING MOIETIES

The chimeric proteins of the invention are composed
of a cell-specific targeting moiety and an apoptosis-inducing
moiety. The cell-specific targeting moiety confers cell-type
30 specific binding to the molecule, and it is chosen on the
basis of the particular cell population to be targeted. A
wide variety of proteins are suitable for use as cell-
specific targeting moieties, including but not limited to,
ligands for receptors such as growth factors, hormones and
35 cytokines, and antibodies or antigen-binding fragments
thereof.

Since a large number of cell surface receptors have been
identified in hematopoietic cells of various lineages,
ligands or antibodies specific for these receptors may be
used as cell-specific targeting moieties. In a specific
5 embodiment illustrated by working examples in Section 6,
infra, IL2 was used as the cell-specific targeting moiety in
a chimeric protein to target IL2R$^+$ cells. In addition, other
molecules such as B7-1, B7-2 and CD40 may be used to
specifically target activated T cells (The Leucocyte Antigen
10 Facts Book, 1993, Barclay et al. (eds.), Academic Press). On
the other hand, B cells express CD19, CD40 and IL4 receptor
and may be targeted by moieties that bind these receptors.
Examples of such moieties include CD40 ligand, IL4, IL5, IL6
and CD28. The elimination of immune cells such as T cells
15 and B cells is particularly useful in the treatment of
autoimmunity, hypersensitivity, transplantation rejection
responses and in the treatment of lymphoid tumors. Examples
of autoimmune diseases are multiple sclerosis, rheumatoid
arthritis, insulin-dependent diabetes mellitus, systemic
20 lupus erythematosis, scleroderma, uviatis, and the like.
More specifically, since myelin basic protein is known to be
the major target of immune cell attack in multiple sclerosis,
this protein may be used as a cell-specific targeting moiety
for the treatment of multiple sclerosis (WO 97/19179; Becker
25 et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:10873).

Other cytokines which may be used to target specific
cell subsets include the interleukins (IL1-IL15),
granulocyte-colony stimulating factor, macrophage-colony
stimulating factor, granulocyte-macrophage colony stimulating
30 factor, leukemia inhibitory factor, tumor necrosis factor,
transforming growth factor, epidermal growth factor, insulin-
like growth factors, fibroblast growth factor and the like
(Thompson (ed.), 1994, The Cytokine Handbook, Academic Press,
San Diego).

35 Additionally, certain cell surface molecules are highly
expressed in tumor cells, including hormone receptors such as
human chorionic gonadotropin receptor and gonadotropin

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releasing hormone receptor (Nechushtan et al., 1997, J. Biol.
Chem. 272:11597). Therefore, the corresponding hormones may
be used as the cell-specific targeting moieties in cancer
therapy.

5   Antibodies are the most versatile cell-specific
targeting moieties because they can be generated against any
cell surface antigen of interest. Monoclonal antibodies have
been generated against cell surface receptors, tumor-
associated antigens, and leukocyte lineage-specific markers

10 such as CD antigens. Antibody variable region genes can be
readily isolated from hybridoma cells by methods well known
in the art. However, since antibodies are assembled between
two heavy chains and two light chains, it is preferred that a
scFv be used as a cell-specific targeting moiety in the

15 present invention. Such scFv are comprised of V_H and V_L
domains linked into a single polypeptide chain by a flexible
linker peptide. Furthermore, the Fc portion of the heavy
chain of an antibody may be used to target Fc receptor-
expressing cells such as the use of the Fc portion of an IgE

20 antibody to target mast cells and basophils. The specific
targeting of these cell types is useful for treating IgE-
mediated hypersensitivity in humans and animals (Helm et al.,
1988, Nature 331:180-183; PCT/IL96/00181).

25   5.1.2.   APOPTOSIS-INDUCING MOIETIES
        The pro-apoptotic proteins in the BCL2 family are
particularly suitable for use as the apoptosis-inducing
moieties in the present invention. Such human proteins are
expected to have reduced immunogenicity over many

30 immunotoxins composed of bacterial toxins. In a specific
embodiment illustrated by working examples in Section 6,
infra, the bax coding sequence is fused with an IL2 coding
sequence for the production of a chimeric protein IL2-Bax.
While Bax is the preferred apoptosis-inducing moiety, other

35 members in this family suitable for use in the present
invention include Bak (Farrow et al., 1995, Nature 374:731;
Chittenden et al., 1995, Nature 374:733; Kiefer et al., 1995,

Nature 374:736), Bcl-X$_s$ (Boise et al., 1993, Cell 74:597; Fang
et al., 1994, J.Immunol. 153:4388), Bad (Yang et al., 1995,
Cell 80:285), Bid (Wang et al., 1996, Genes Develop. 10:2859-
2869), Bik (Bovd et al., 1995, Oncogene 11:1921-1928), Hrk
5   (Inohara et al., 1997, EMBO J. 16:1686-1694) and Bok (Hsu et
al., 1997, Proc. Natl. Acad. Sci. USA 94: 12401-12406). The
nucleotide sequences encoding these proteins are known in the
art, and thus cDNA clones can be readily obtained for fusion
with a coding sequence for a cell-specific targeting moiety
10 in an expression vector.

        Specific domains of certain of the Bcl-2 family members
have been studied with respect to their apoptosis-inducing
activities. For example, the GD domain of Bak is involved in
the apoptosis function (U.S. Patent No. 5,656,725). In
15 addition, Bax and Bip1a are shown to share a homologous
domain. Therefore, any biologically active domains of the
Bcl-2 family may be used as an apoptosis-inducing moiety for
the practice of the present invention.

        Caspases also play a central role in apoptosis and may
20 well constitute part of the consensus core mechanism of
apoptosis. Caspases are implicated as mediators of
apoptosis. Since the recognition that CED-3, a protein
required for developmental cell death, has sequence identity
with the mammalian cysteine protease interleukin-1 beta-
25 converting enzyme (ICE), a family of at least 10 related
cysteine proteases has been identified. These proteins are
characterized by almost absolute specificity for aspartic
acid in the P1 position. All the caspases (ICE-like
proteases) contain a conserved QACXG (where X is R, Z or G)
30 pentapeptide active-site motif. Caspases are synthesized as
inactive proenzymes comprising an N-terminal peptide
(Prodomain) together with one large and one small subunit.
The crystal structures of both caspase-1 and caspase-3 show
that the active enzyme is a heterotetramer, containing two
35 small and two large subunits. Activation of caspases during
apoptosis results in the cleavage of critical cellular
substrates, including poly(ADP-ribose) polymerase and lamins,

so precipitating the dramatic morphological changes of apoptosis (Cohen, 1997, Biochem. J. 326:1-16). Therefore, it is also within the scope of the present invention to use a caspase as an apoptosis-inducing moiety.

5   Recently a few new proteins were cloned and identified as factors required for mediating activity of proteins, mainly caspases, involved in the apoptosis pathway. One factor was identified as the previously known electron transfer protein, cytochrome c (Lin et al., 1996, Cell

10 86:147-157), designed as Apaf-2. In addition to cytochrome c the activation of caspase-3 requires two other cytosolic factors-Apaf-1 and Apaf-3. Apaf-1 is a protein homologous to C. elegans CED-4, and Apaf-3 was identified as a member of the caspase family, caspase-9. Both factors bind to each

15 other via their respective NH2-terminal CED-3 homologous domains, in the presence of cytochrome c, an event that leads to caspase-9 activation. Activated caspase-9 in turn cleaves and activates caspase-3 (Liu et al., 1996, Cell 86:147-157; Zou et al., 1997, Cell 90:405-413; Li et al., 1997, Cell

20 91:479-489). Another protein involved in the apoptotic pathway is DNA fragmentation factor (DFF), a heterodimer of 45 and 40 kd subunits that functions downstream of caspase-3 to trigger fragmentation of genomic DNA into nucleosomal segments (Liu et al., 1997, Cell 89:175-184).

25

5.2. EXPRESSION OF CHIMERIC PROTEINS

In accordance with the invention, a polynucleotide which encodes a chimeric protein, mutant polypeptides, biologically active fragments of chimeric protein, or functional

30 equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of the chimeric protein, chimeric peptide fragments, or a functional equivalent thereof, in appropriate host cells.

Due to the inherent degeneracy of the genetic code,

35 other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression

of the chimeric protein. Such DNA sequences include those
which are capable of hybridizing to the chimeric sequences or
their complementary sequences under stringent conditions.
The phrase "stringent conditions" as used herein refers to
5 those hybridizing conditions that (1) employ low ionic
strength and high temperature for washing, for example, 0.015
M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.; (2) employ
during hybridization a denaturing agent such as formamide,
for example, 50% (vol/vol) formamide with 0.1% bovine serum
10 albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium
phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium
citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M
NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution,
sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10%
15 dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and
0.1% SDS.

Altered DNA sequences which may be used in accordance
with the invention include deletions, additions or
substitutions of different nucleotide residues resulting in a
20 sequence that encodes the same or a functionally equivalent
fusion gene product. The gene product itself may contain
deletions, additions or substitutions of amino acid residues
within a chimeric sequence, which result in a silent change
thus producing a functionally equivalent chimeric protein.
25 Such amino acid substitutions may be made on the basis of
similarity in polarity, charge, solubility, hydrophobicity,
hydrophilicity, and/or the amphipathic nature of the residues
involved. For example, negatively charged amino acids
include aspartic acid and glutamic acid; positively charged
30 amino acids include lysine, histidine and arginine; amino
acids with uncharged polar head groups having similar
hydrophilicity values include the following: glycine,
asparagine, glutamine, serine, threonine, tyrosine; and amino
acids with nonpolar head groups include alanine, valine,
35 isoleucine, leucine, phenylalanine, proline, methionine,
tryptophan.

The DNA sequences of the invention may be engineered in
order to alter a chimeric coding sequence for a variety of
ends, including but not limited to, alterations which modify
processing and expression of the gene product. For example,
5 mutations may be introduced using techniques which are well
known in the art, e.g., site-directed mutagenesis, to insert
new restriction sites, to alter glycosylation patterns,
phosphorylation, etc.

In an alternate embodiment of the invention, the coding
10 sequence of the chimeric protein could be synthesized in
whole or in part, using chemical methods well known in the
art. See, for example, Caruthers et al., 1980, Nuc. Acids
Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids
Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron
15 Letter 21:719; and Chow and Kempe, 1981, Nuc. Acids Res.
9(12):2807-2817. For example, active domains of the moieties
can be synthesized by solid phase techniques, cleaved from
the resin, and purified by preparative high performance
liquid chromatography followed by chemical linkage to form a
20 chimeric protein. (e.g., see Creighton, 1983, Proteins
Structures And Molecular Principles, W.H. Freeman and Co.,
N.Y. pp. 50-60). The composition of the synthetic peptides
may be confirmed by amino acid analysis or sequencing (e.g.,
the Edman degradation procedure; see Creighton, 1983,
25 Proteins, Structures and Molecular Principles, W.H. Freeman
and Co., N.Y., pp. 34-49). Alternatively, the two moieties
of the chimeric protein produced by synthetic or recombinant
methods may be conjugated by chemical linkers according to
methods well known in the art (Brinkmann and Pastan, 1994,
30 Biochemica et Biophysica Acta 1198:27-45).

In order to express a biologically active chimeric
protein, the nucleotide sequence coding for a chimeric
protein, or a functional equivalent, is inserted into an
appropriate expression vector, i.e., a vector which contains
35 the necessary elements for the transcription and translation
of the inserted coding sequence. The chimeric gene products
as well as host cells or cell lines transfected or

transformed with recombinant chimeric expression vectors can
be used for a variety of purposes. These include but are not
limited to generating antibodies (*i.e.*, monoclonal or
polyclonal) that bind to epitopes of the proteins to
5 facilitate their purification.

Methods which are well known to those skilled in the art
can be used to construct expression vectors containing the
chimeric protein coding sequence and appropriate
transcriptional/translational control signals. These methods
10 include *in vitro* recombinant DNA techniques, synthetic
techniques and *in vivo* recombination/genetic recombination.
See, for example, the techniques described in Sambrook et
al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring
Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current
15 Protocols in Molecular Biology, Greene Publishing Associates
and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be
utilized to express the chimeric protein coding sequence.
These include but are not limited to microorganisms such as
20 bacteria transformed with recombinant bacteriophage DNA,
plasmid DNA or cosmid DNA expression vectors containing the
chimeric protein coding sequence; yeast transformed with
recombinant yeast expression vectors containing the chimeric
protein coding sequence; insect cell systems infected with
25 recombinant virus expression vectors (e.g., baculovirus)
containing the chimeric protein coding sequence; plant cell
systems infected with recombinant virus expression vectors
(e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus,
TMV) or transformed with recombinant plasmid expression
30 vectors (e.g., Ti plasmid) containing the chimeric protein
coding sequence; or animal cell systems. It should be noted
that since most apoptosis-inducing proteins cause programmed
cell death in mammalian cells, it is preferred that the
chimeric protein of the invention be expressed in prokaryotic
35 or lower eukaryotic cells. Section 6 illustrates that IL2-
Bax may be efficiently expressed in *E. coli*.

The expression elements of each system vary in their
strength and specificities. Depending on the host/vector
system utilized, any of a number of suitable transcription
and translation elements, including constitutive and
5 inducible promoters, may be used in the expression vector.
For example, when cloning in bacterial systems, inducible
promoters such as pL of bacteriophage λ, plac, ptrp, ptac
(ptrp-lac hybrid promoter; cytomegalovirus promoter) and the
like may be used; when cloning in insect cell systems,
10 promoters such as the baculovirus polyhedrin promoter may be
used; when cloning in plant cell systems, promoters derived
from the genome of plant cells (e.g., heat shock promoters;
the promoter for the small subunit of RUBISCO; the promoter
for the chlorophyll α/β binding protein) or from plant
15 viruses (e.g., the 35S RNA promoter of CaMV; the coat protein
promoter of TMV) may be used; when cloning in mammalian cell
systems, promoters derived from the genome of mammalian cells
(e.g., metallothionein promoter) or from mammalian viruses
(e.g., the adenovirus late promoter; the vaccinia virus 7.5K
20 promoter) may be used; when generating cell lines that
contain multiple copies of the chimeric DNA, SV40-, BPV- and
EBV-based vectors may be used with an appropriate selectable
marker.

In bacterial systems a number of expression vectors may
25 be advantageously selected depending upon the use intended
for the chimeric protein expressed. For example, when large
quantities of chimeric protein are to be produced, vectors
which direct the expression of high levels of protein
products that are readily purified may be desirable. Such
30 vectors include but are not limited to the pHL906 vector
(Fishman et al., 1994, Biochem. 33:6235-6243), the *E. coli*
expression vector pUR278 (Ruther et al., 1983, EMBO J.
2:1791), in which the chimeric protein coding sequence may be
ligated into the vector in frame with the *lacZ* coding region
35 so that a hybrid AS-*lacZ* protein is produced; pIN vectors
(Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van

Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and
the like.

   An alternative expression system which could be used to
express chimeric protein is an insect system. In one such
5 system, *Autographa californica* nuclear polyhidrosis virus
(AcNPV) is used as a vector to express foreign genes. The
virus grows in *Spodoptera frugiperda* cells. The chimeric
protein coding sequence may be cloned into non-essential
regions (for example the polyhedrin gene) of the virus and
10 placed under control of an AcNPV promoter (for example the
polyhedrin promoter). Successful insertion of the chimeric
protein coding sequence will result in inactivation of the
polyhedrin gene and production of non-occluded recombinant
virus (i.e., virus lacking the proteinaceous coat coded for
15 by the polyhedrin gene). These recombinant viruses are then
used to infect *Spodoptera frugiperda* cells in which the
inserted gene is expressed. (e.g., see Smith et al., 1983,
J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

   Specific initiation signals may also be required for
20 efficient translation of the inserted chimeric protein coding
sequence. These signals include the ATG initiation codon and
adjacent sequences. In cases where the entire chimeric gene,
including its own initiation codon and adjacent sequences, is
inserted into the appropriate expression vector, no
25 additional translational control signals may be needed.
However, in cases where the chimeric protein coding sequence
does not include its own initiation codon, exogenous
translational control signals, including the ATG initiation
codon, must be provided. Furthermore, the initiation codon
30 must be in phase with the reading frame of the chimeric
protein coding sequence to ensure translation of the entire
insert. These exogenous translational control signals and
initiation codons can be of a variety of origins, both
natural and synthetic. The efficiency of expression may be
35 enhanced by the inclusion of appropriate transcription
enhancer elements, transcription terminators, etc. (see
Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which
modulates the expression of the inserted sequences, or
modifies and processes the gene product in the specific
fashion desired. Such modifications (e.g., glycosylation)
5 and processing (e.g., cleavage) of protein products may be
important for the function of the protein. The presence of
consensus N-glycosylation sites in a chimeric protein may
require proper modification for optimal chimeric protein
function. Different host cells have characteristic and
10 specific mechanisms for the post-translational processing and
modification of proteins. Appropriate cell lines or host
systems can be chosen to ensure the correct modification and
processing of the chimeric protein. To this end, eukaryotic
host cells which possess the cellular machinery for proper
15 processing of the primary transcript, glycosylation, and
phosphorylation of the chimeric protein may be used. Such
mammalian host cells include but are not limited to CHO,
VERO, BHK, HeLa, COS, MDCK, 293, WI38, and the like.
    For long-term, high-yield production of recombinant
20 chimeric proteins, stable expression is preferred. For
example, cell lines which stably express the chimeric protein
may be engineered. Rather than using expression vectors
which contain viral origins of replication, host cells can be
transformed with a chimeric coding sequence controlled by
25 appropriate expression control elements (e.g., promoter,
enhancer, sequences, transcription terminators,
polyadenylation sites, etc.), and a selectable marker.
Following the introduction of foreign DNA, engineered cells
may be allowed to grow for 1-2 days in an enriched media, and
30 then are switched to a selective media. The selectable
marker in the recombinant plasmid confers resistance to the
selection and allows cells to stably integrate the plasmid
into their chromosomes and grow to form foci which in turn
can be cloned and expanded into cell lines.
35   A number of selection systems may be used, including but
not limited to the herpes simplex virus thymidine kinase
(Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine

phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells,

5 respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid

10 (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and *hygro*, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147) genes. Additional selectable genes

15 have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and *ODC* (ornithine decarboxylase) which confers resistance to the

20 ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

25   5.3. **PROTEIN PURIFICATION**
        The chimeric proteins of the invention can be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography and the like. The

30 actual conditions used to purify a particular protein will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, etc., and will be apparent to those having skill in the art.
        For affinity chromatography purification, any antibody

35 which specifically binds the protein may be used. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by

injection with a chimeric protein or a fragment thereof. The
protein may be attached to a suitable carrier, such as bovine
serum albumin (BSA), by means of a side chain functional
group or linkers attached to a side chain functional group.
5 Various adjuvants may be used to increase the immunological
response, depending on the host species, including but not
limited to, Freund's (complete and incomplete), mineral gels
such as aluminum hydroxide, surface active substances such as
lysolecithin, pluronic polyols, polyanions, peptides, oil
10 emulsions, keyhole limpet hemocyanin, dinitrophenol, and
potentially useful human adjuvants such as BCG (bacilli
Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to a chimeric protein may be
prepared using any technique which provides for the
15 production of antibody molecules by continuous cell lines in
culture. These include but are not limited to the hybridoma
technique originally described by Koehler and Milstein (1975,
Nature 256:495-497), the human B-cell hybridoma technique,
(Kosbor et al., 1983, Immunology Today 4:72; Cote et al.,
20 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) and the
EBV-hybridoma technique (Cole et al., 1985, Monoclonal
Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-
96). In addition, techniques developed for the production of
"chimeric antibodies" (Morrison et al., 1984, Proc. Natl.
25 Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984,
Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454)
by splicing the genes from a mouse antibody molecule of
appropriate antigen specificity together with genes from a
human antibody molecule of appropriate biological activity
30 can be used. Alternatively, techniques described for the
production of single chain antibodies (U.S. Patent No.
4,946,778) can be adapted to produce chimeric protein-
specific single chain antibodies for chimeric protein
purification and detection.
35

5.4. USES OF CHIMERIC PROTEINS

Once a chimeric protein is expressed and purified, its
identity and functional activities can be readily determined
by methods well known in the art. For example, antibodies to
5 the two moieties of the protein may be used to identify the
protein in Western blot analysis. In addition, the chimeric
protein can be tested for specific binding to target cells in
binding assays using a fluorescent-labeled or radiolabelled
secondary antibody.
10

5.4.1.   *IN VITRO AND EX VIVO USES*

The chimeric proteins of the invention are useful
for targeting specific cell types in a cell mixture, and
eliminating the target cells by inducing apoptosis. For
15 example, IL2-Bax may be used to purge IL2R⁺ leukemic cells in
a bone marrow preparation or mobilized peripheral blood prior
to infusion of the cells into a recipient following ablative
therapy. In addition, this chimeric protein may be used to
deplete IL2R⁺ cells in a donor cell preparation prior to
20 allogeneic or xenogeneic bone marrow transplantation in order
to reduce the development of graft-versus-host disease. It
can also be used for *ex vivo* purging of specific cell subsets
in any body fluids such as cerebral spinal fluid, pleural
fluid and sinovial fluid.
25   The chimeric protein of the invention is also useful as
a diagnostic reagent. For example, IL2-Bax may be used to
detect the presence of autoimmune IL2R-expressing cells in a
body fluid or to detect the tissue origin of an IL2R⁺
lymphoma. The binding of a chimeric protein to a target cell
30 can be readily detected by using a secondary antibody
specific for the apoptosis-inducing moiety. In that
connection, the secondary antibody or the chimeric protein
can be linked to a detectable label such as fluorescein, an
enzyme or a radioisotope to facilitate the detection of
35 binding of the chimeric protein to a cell.

5.4.2.   *IN VIVO USES*

The chimeric proteins of the invention may be administered to a subject *per se* or in the form of a pharmaceutical composition for the treatment of cancer,

5 autoimmunity, transplantation rejection, post-traumatic immune responses and infectious diseases by targeting viral antigens such as gp120 of HIV. More specifically, IL2-Bax is useful for eliminating activated IL2R⁺ cells involved in immune cell-mediated disorder, including lymphoma;

10 autoimmunity, transplantation rejection, graft-versus-host disease, ischemia and stroke. Pharmaceutical compositions comprising the proteins of the invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating,

15 entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the proteins into preparations which can be used

20 pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration the proteins of the invention may be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.

25 Systemic formulations include those designed for administration by injection, *e.g.* subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, inhalation, oral or pulmonary administration.

30 For injection, the proteins of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending,

35 stabilizing and/or dispersing agents.

Alternatively, the proteins may be in powder form for
constitution with a suitable vehicle, *e.g.*, sterile pyrogen-
free water, before use.

For transmucosal administration, penetrants appropriate
5 to the barrier to be permeated are used in the formulation.
Such penetrants are generally known in the art.

For oral administration, the proteins can be readily
formulated by combining the proteins with pharmaceutically
acceptable carriers well known in the art. Such carriers
10 enable the proteins of the invention to be formulated as
tablets, pills, dragees, capsules, liquids, gels, syrups,
slurries, suspensions and the like, for oral ingestion by a
patient to be treated. For oral solid formulations such as,
for example, powders, capsules and tablets, suitable
15 excipients include fillers such as sugars, e.g. lactose,
sucrose, mannitol and sorbitol; cellulose preparations such
as maize starch, wheat starch, rice starch, potato starch,
gelatin, gum tragacanth, methyl cellulose,
hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose,
20 and/or polyvinylpyrrolidone (PVP); granulating agents; and
binding agents. If desired, disintegrating agents may be
added, such as the cross-linked polyvinylpyrrolidone, agar,
or alginic acid or a salt thereof such as sodium alginate.

If desired, solid dosage forms may be sugar-coated or
25 enteric-coated using standard techniques.

For oral liquid preparations such as, for example,
suspensions, elixirs and solutions, suitable carriers,
excipients or diluents include water, glycols, oils,
alcohols, etc. Additionally, flavoring agents,
30 preservatives, coloring agents and the like may be added.

For buccal administration, the proteins may take the
form of tablets, lozenges, etc. formulated in conventional
manner.

For administration by inhalation, the proteins for use
35 according to the present invention are conveniently delivered
in the form of an aerosol spray from pressurized packs or a
nebulizer, with the use of a suitable propellant, e.g.,

- 28 -

dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered

5　amount. Capsules and cartridges of gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the protein and a suitable powder base such as lactose or starch.

　　　The proteins may also be formulated in rectal or vaginal

10　compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

　　　In addition to the formulations described previously, the proteins may also be formulated as a depot preparation.

15　Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an

20　acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

　　　Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well known examples

25　of delivery vehicles that may be used to deliver proteins of the invention. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the proteins may be delivered using a sustained-release system, such as

30　semipermeable matrices of solid polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the proteins for a few weeks up to

35　over 100 days. Depending on the chemical nature and the biological stability of the chimeric protein, additional strategies for protein stabilization may be employed.

As the proteins of the invention may contain charged
side chains or termini, they may be included in any of the
above-described formulations as the free acids or bases or as
pharmaceutically acceptable salts. Pharmaceutically
5 acceptable salts are those salts which substantially retain
the biologic activity of the free bases and which are
prepared by reaction with inorganic acids. Pharmaceutical
salts tend to be more soluble in aqueous and other protic
solvents than are the corresponding free base forms.
10

5.4.3. EFFECTIVE DOSAGES

The proteins of the invention will generally be
used in an amount effective to achieve the intended purpose.
For use to treat or prevent a disease condition, the proteins
15 of the invention, or pharmaceutical compositions thereof, are
administered or applied in a therapeutically effective
amount. A therapeutically effective amount is an amount
effective to ameliorate or prevent the symptoms, or prolong
the survival of, the patient being treated. Determination of
20 a therapeutically effective amount is well within the
capabilities of those skilled in the art, especially in light
of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective
dose can be estimated initially from *in vitro* assays. For
25 example, a dose can be formulated in animal models to achieve
a circulating concentration range that includes the IC_{50} as
determined in cell culture. Such information can be used to
more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data,
30 *e.g.*, animal models, using techniques that are well known in
the art. One having ordinary skill in the art could readily
optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually
to provide plasma levels of the proteins which are sufficient
35 to maintain therapeutic effect. Usual patient dosages for
administration by injection range from about 0.1 to 5
mg/kg/day, preferably from about 0.5 to 1 mg/kg/day.

Therapeutically effective serum levels may be achieved by
administering multiple doses each day.

In cases of local administration or selective uptake,
the effective local concentration of the proteins may not be
5 related to plasma concentration. One having skill in the art
will be able to optimize therapeutically effective local
dosages without undue experimentation.

The amount of protein administered will, of course, be
dependent on the subject being treated, on the subject's
10 weight, the severity of the affliction, the manner of
administration and the judgment of the prescribing physician.

The therapy may be repeated intermittently while
symptoms detectable or even when they are not detectable.
The therapy may be provided alone or in combination with
15 other drugs. In the case of autoimmune disorders, the drugs
that may be used in combination with IL2-Bax of the invention
include, but are not limited to, steroid and non-steroid
anti-inflammatory agents.

20   5.4.4. **TOXICITY**
Preferably, a therapeutically effective dose of the
chimeric proteins described herein will provide therapeutic
benefit without causing substantial toxicity.

Toxicity of the proteins described herein can be
25 determined by standard pharmaceutical procedures in cell
cultures or experimental animals, e.g., by determining the
LD_{50} (the dose lethal to 50% of the population) or the LD_{100}
(the dose lethal to 100% of the population). The dose ratio
between toxic and therapeutic effect is the therapeutic
30 index. Proteins which exhibit high therapeutic indices are
preferred. The data obtained from these cell culture assays
and animal studies can be used in formulating a dosage range
that is not toxic for use in human. The dosage of the
proteins described herein lies preferably within a range of
35 circulating concentrations that include the effective dose
with little or no toxicity. The dosage may vary within this
range depending upon the dosage form employed and the route

of administration utilized. The exact formulation, route of
administration and dosage can be chosen by the individual
physician in view of the patient's condition. (*See, e.g.,*
Fingl *et al.*, 1975, In: The Pharmacological Basis of
5 Therapeutics, Ch.1, p.1).

The invention having been described, the following
examples are offered by way of illustration and not
limitation.

10 6.   **EXAMPLE: PRODUCTION OF A CHIMERIC PROTEIN THAT
              INDUCED IL2R⁺ CELL-SPECIFIC APOPTOSIS**

6.1. MATERIALS AND METHODS

6.1.1.   CONSTRUCTION OF IL2-BAX CODING SEQUENCE

A plasmid for the expression of IL2-Bax chimeric
15 protein under the control of the T7 promoter was constructed
as shown in Figure 1. pHL906 which carried the fusion gene
IL2-PE40 was cut with HindIII and PpuMI to remove the PE
sequence, and the vector fragment was eluted (Fishman et al.,
1994, Biochem. 33:6235-6243). A cDNA encoding·human Bax-α
20 was obtained by reverse transcription-polymerase chain
reaction (RT-PCR), using RNA isolated from fresh human
lymphocytes. Total RNA was isolated and was reverse
transcribed into first strand cDNA, using the reverse
transcription system (Promega, USA) under conditions
25 recommended by the manufacturer. The cDNA was diluted to a
total volume of 1 ml with 10 mM Tris-HCl pH 7.6, 1 mM EDTA
and stored at 4°C. ·The Bax-encoding fragment was generated
by PCR using this cDNA and a pair of synthetic
oligonucleotide primers: 5' CGCAATTCAAGCTTTGGACGGGTCCGGGGGA
30 3' (SEQ ID NO:3) (sense) and
5' CGGAATTCAGGTCGTTCAGCCCATCTTCTTC 3' (SEQ ID NO:4)
(antisense) covering the entire coding region. The reaction
mixture was incubated in a DNA thermal cycler (MJ Research
Inc., Watertown, MA) for 33 cycles. Each cycle consisted of
35 1 min. at 95°C, 1 min. at 65°C and 2 min. at 72°C. The Bax-
encoding fragment was digested with EcoRI and HindIII enzymes
and ligated with the pHL906 vector. The resulting plasmid,

designated pSY1, contained the human IL2 coding sequence
fused to the 5' end of the human Bax coding sequence. The
plasmid was confirmed by restriction endonuclease digestion
and DNA sequence analysis. The nucleotide and deduced amino
5 acid sequences (SEQ ID NOS:1 and 2) of the chimeric molecule
referred to as IL2-Bax are disclosed in Figure 2.

6.1.2.   PROTEIN EXPRESSION AND
PARTIAL PURIFICATION

The pSY1 plasmid containing the fused coding
10 sequences was transformed into *E. coli* strain BL21 (λDE3) and
the IL2-Bax chimeric protein was expressed. A pellet of
expressing cells was suspended in 50 mM Tris-HCl pH 8.0, 1 mM
EDTA containing 0.2 mg/ml lysozyme, sonicated (three 30-s
bursts) and centrifuged at 30,000 Xg for 30 min. The
15 supernatant (soluble fraction) was removed and kept for
analysis. The pellet was denatured in one of three
extraction buffers:

1) Extraction buffer A: 6 M Guanidine-HCl, 0.1 M Tris-
HCl pH 8.6, 1 mM EDTA, 0.05 M NaCl, and 10 mM DTT, and
20 stirred for 30 min. at 4°C. The suspension was cleared by
centrifugation at 30,000 Xg for 15 min. and the pellet
discarded. The protein solution was diluted 1:100 in
refolding buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M
NaCl, 0.25 M L-arginine, and 5 mM dithiothreitol) and kept at
25 4°C for 48 h. The refolded protein solution was dialyzed
against phosphate-buffered saline (PBS).

2) Extraction buffer B: 20 mM Tris-HCl pH 7.4, 150 mM
NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid, 0.1% SDS.
Before testing its activity, the fraction was dialyzed
30 against PBS.

3) Extraction buffer C: 8 M Urea, 50 mM Tris HCl pH 8.0,
1 mM EDTA, 10 mM DTT (v/w 1:1) for 1 hr, centrifuged at
35,000 xg for 15 min. The supernatant was diluted 1:100 with
refolding buffer (see above) without dithiothreitol.

35 The protein profile of various fractions (soluble
fraction, insoluble fraction-treated in three different

protocols) were characterized by gel electrophoresis (Figure 3).

6.1.3. **WESTERN BLOT ANALYSIS**

5   The electrophoresis samples were transferred onto nitrocellulose and immunoblotted as described (Fishman and Lorberboum-Galski, 1997, Eur. J. Immunol. 27:486-494). The ECL detection kit (Amersham, Bukinghamshire, UK) was used according to the manufacturer's instructions. A protein

10 extract from MCF-7 cells (breast carcinoma-cell line), known to express the Bax protein, was used as a positive control. Anti-human Bax was obtained from Pharmingen (San Diego, California) and used at a dilution of 1:2,500. Anti-human IL2 was obtained from Endogen and used at a dilution of

15 1:5,000.

6.1.4. **ASSAY FOR IDENTIFYING APOPTOTIC CELLS**

        Human peripheral blood lymphocytes from healthy donors were separated using Ficoll-Isopaque gradient (1.077)

20 (Pharmacia) and used immediately. Lymphocytes were cultured in 5% CO_2 in air in RPMI 1640 medium supplemented with 10% fetal calf serum, 200 μg/ml L-glutamate, 50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml glutamine and 5 X 10^{-5} M 2-β mercaptoethanol. Increasing concentrations of IL2-Bax

25 (insoluble fraction, 6M Gu-HCl treated) were added to the lymphocytes for 22 hr. Cells were then stained with:

        A. Propidium Iodide (PI, 3.5 μg/ml)

        B. Propidium Iodide with a detergent for measuring cell cycle (0.7 ml of the PI buffer: 50 μg/ml PI, 0.1% Na-

30 citrate, 0.1% Triton x100, were added to a cell pellet of ~10^{6} cells). Cells were then analyzed by FACS.

        HUT102 cells (target T cells) and CEM cells (non-target T cells) were incubated overnight with increasing concentrations of IL2-Bax. The 200 xg centrifuged cell

35 pellet was fixed in 2 ml cold 70% ethanol at 4°C for 60 min. The cells were then centrifuged, washed in 1 ml PBS and resuspended in 0.5 ml PBS. 0.5 ml RNAse (Type I-A, Sigma,

- 34 -

St. Louis, Missouri, 1 mg/ml in PBS) was added to the sample,
followed by gentle mixing with 1 ml PI (Sigma, 100 μg/ml PBS)
solution. The mixed cells were incubated in the dark at room
temperature for 15 min. and kept at 4°C in the dark until
5 measured. The PI fluorescence of the individual nuclei was
measured using FACS flow cytometer. The forward scatter and
side scatter of particles were simultaneously measured. Cell
debris were excluded from analysis by appropriately raising
the forward scatter threshold (Nicoletti et al., 1991, J.
10 Immunol. Meth. 139:271-279).

6.1.5.   CYTOTOXICITY ASSAY

Cells (10^4 in 0.2 ml culture medium) were seeded in
96-well microplates, followed by the addition of various
15 concentrations of the chimeric protein (diluted with 0.25%
BSA in PBS). After a 24 hour incubation, [^{3}H]leucine (2-
5uci/well) were added for 6-13 hr. The plates were then
stored at -70°C for several hours, followed by quick thawing
at 37°C. This step was omitted with targets cells growing in
20 suspension. The cells were harvested on filters and the
incorporated radioactivity was measured with a β counter.
Results were expressed as the percent incorporation of the
control experiments in which the cells were not exposed to
any protein. All assays were carried out in triplicates.
25

6.2. RESULTS

An expression plasmid encoding an IL2-Bax chimeric
protein was constructed under the control of the T7 promoter.
The plasmid was expressed in *E. coli* and the chimeric protein
30 was extracted. The protein was further characterized by
Western blot analysis using antibodies against Bax and IL2
(Figure 4). The chimeric protein reacted with the antibodies
to Bax and to IL2, confirming the cloning and production of
in-frame full-length IL2-Bax chimeric protein.
35   The cytotoxic activity of the IL2-Bax chimeric protein
was tested on HUT102 and MT-1 cells (human T-cell lines), and
2B4 cells (mouse T-cells); all known to express the high

affinity receptor for IL2, by a quantitative assay, in which
inhibition of protein synthesis was measured. The soluble
and insoluble fractions generated from treatment under three
different conditions were tested in cytotoxicity assays as
5 described by Lorberboum-Galski et al. (1988, J. Biol. Chem.
263:18650-18656). As shown in Figure 5A, all three IL2R-
expressing cell lines were responsive to the chimeric protein
in a dose dependent manner, although with different
sensitivities. This may be attributed to the different
10 number of IL2R expressed on each cell line. The insoluble
fraction treated with ether extraction buffer containing Gu-
HCl or SDS exhibited the highest activity toward the various
cells. Therefore, further experiments were performed with
mainly the partially purified fractions (insoluble fraction
15 extracted with Gu-HCl or SDS). In all experiments IL2-PE
chimeric proteins, previously shown to be cytotoxic to IL2R⁺
cells, were used as positive control.

The effects of IL2-Bax were also tested on various IL2R
negative cells: CEM cells (a human T-cell line lacking the
20 IL2R) and Km3 (a human non-T, non-B stem cell line). Figure
5B shows that these cell lines were unaffected by the IL2-Bax
chimeric protein. Since the fraction treated with Gu-HCl was
active without any non-specific cytotoxicity, this fraction
was further used in subsequent experiments.

25   The ability of IL2-Bax to induce apoptosis in IL2R⁺ cells
was determined in an apoptosis assay. Figure 6A-6D shows an
increase of apoptotic cells in a freshly isolated lymphocyte
population treated with IL2-Bax, and the effects of the
chimeric protein were dose dependent. The apoptotic cells
30 ranged from 2% to 14% (Table 1A, B) of the total cell
population (Figure 7A-7C). In that regard, it should be
noted that freshly isolated lymphocytes from healthy donors
usually contain only low levels of cells expressing the IL2R,
thus the percentage of cells expected to be targeted by IL2-
35 Bax in fresh lymphocytes is low.

Dexamethasone (10^{-7} M), a known inducer for apoptosis in
various cells, was used in all experiments to follow

apoptosis. However, it is well known that various cells respond differently to, if at all, to this reagent. Dexamethasone was also shown to be a weak inducer of apoptosis in fresh lymphocytes (Figure 6B). 2B4 cells, known
5 to react very strongly to this agent were used as control cells to detect apoptosis. In conclusion, recombinant chimeric protein IL2-Bax was specifically cytotoxic to IL2R⁺ cells, but did not affect IL2R⁻ cells. Furthermore, the cytotoxic effects of the chimeric protein were mediated by an
10 induction of apoptosis, as evidenced by its ability to induce programmed cell death in freshly isolated human lymphocytes.

<div align="center">

Table 1

Effect of IL2-Bax on Fresh Lymphocytes Analyzed by FACS

</div>

15 A. Experiment No. 1

Treatment	M1	M2	M1-2
control	7.1	1.9	5.2
dexamethasone	9.5	2.8	6.7
IL2-Bax, 1 µg	17.5	4	13.5
IL2-Bax, 5 µg	24.3	4.3	20
IL2-Bax, 10 µg	42.1	9.1	35

Each M1 or M2 values are the mean of duplicates.

B. Experiment No. 2

Treatment	UL	UR	UL+UR
control	2.36	2.12	4.48
IL2-Bax, 1 µg	4.98	5.56	10.54
IL2-Bax, 10 µg	14.71	16.84	31.55

Each UL or UR value is the mean of duplicates.
30

Figure 8A-8E demonstrates the increase of an apoptotic-cell population in HUT102 cells exposed to IL2-Bax in a dose dependent manner (M1 values represent the sub-G1 apoptotic-cell population). At the highest concentration tested, IL2-
35 Bax induced a 3.6-fold increase in the percentage of the apoptotic-cell population. In contrast, CEM cells which lacked IL2R expression did not show an increase in the

apoptotic cell population (Figure 9A-9C), confirming the
specificity of the effects of IL2-Bax.

　　　The present invention is not to be limited in scope by
5 the exemplified embodiments which are intended as
illustrations of single aspects of the invention and any
sequences which are functionally equivalent are within the
scope of the invention. Indeed, various modifications of the
invention in addition to those shown and described herein
10 will become apparent to those skilled in the art from the
foregoing description and accompanying drawings. Such
modifications are intended to fall within the scope of the
appended claims.

　　　All publications cited herein are incorporated by
15 reference in their entirety.

20

25

30

35

WHAT IS CLAIMED IS

1.   A chimeric protein comprising a cell-specific targeting moiety and an apoptosis-inducing moiety.

5 2.   The chimeric protein of Claim 1 in which the apoptosis-inducing moiety is a protein of human origin.

3.   The chimeric protein of Claim 2 in which the apoptosis-inducing moiety is a pro-apoptotic member of the BCL2 family

10 or an active domain thereof.

4.   The chimeric protein of Claim 3 in which the apoptosis-inducing moiety is Bax or an active domain thereof.

15 5.   The chimeric protein of Claim 4 in which the active domain is the BH3 domain.

6.   The chimeric protein of Claim 1 in which the apoptosis-inducing moiety is a caspase.

20

7.   The chimeric protein of Claim 1 in which the apoptosis-inducing moiety is cytochrome C.

8.   The chimeric protein of Claim 1 in which the cell-

25 specific targeting moiety binds interleukin 2 receptor-expressing cells.

9.   The chimeric protein of Claim 1 in which the cell-specific targeting moiety is an interleukin.

30

10.   The chimeric protein of Claim 9 in which the cell-specific targeting moiety is an interleukin 2.

11.   The chimeric protein of Claim 1 in which the cell-

35 specific targeting moiety is interleukin 2 and the apoptosis-inducing moiety is Bax-α.

12. The chimeric protein of Claim 1 in which the cell-specific targeting moiety is myelin basic protein.

13. The chimeric protein of Claim 1 in which the cell-specific targeting moiety binds the tumor cells.

14. The chimeric protein of Claim 13 in which the cell-specific targeting moiety is gonadotropin releasing hormone.

15. The chimeric protein of Claim 1 in which the cell-specific targeting moiety is an antibody or a fragment thereof.

16. The chimeric protein of Claim 15 in which the cell-specific targeting moiety is a single chain antibody.

17. The chimeric protein of Claim 15 in which the cell-specific targeting moiety is a Fc fragment of an IgE antibody.

18. The chimeric protein of Claim 1 in which the cell-specific targeting moiety is a cytokine.

19. The chimeric protein of Claim 18 in which the cell-specific targeting moiety is epidermal growth factor.

20. The chimeric protein of Claim 18 in which the cell-specific targeting moiety is insulin-like growth factor.

21. The chimeric protein of Claim 1 which is produced by a recombinant DNA method.

22. The chimeric protein of Claim 1 which is produced by a chemical conjugation method.

23. The chimeric protein of Claim 1 in which the two moieties are connected by a polylinker.

24. A pharmaceutical composition comprising a chimeric protein of any of Claims 1-22.

25. A method of inhibiting growth of an interleukin 2
5 receptor-expressing cell, comprising administering an effective amount of a chimeric protein which comprises an interleukin 2 receptor-expressing cell-specific targeting moiety and an apoptosis-inducing moiety.

10 26. A method of treating an autoimmune disease, comprising administering a therapeutically effective amount of a chimeric protein which comprises an interleukin 2 receptor targeting moiety and an apoptosis-inducing moiety.

15 27. The method of Claim 25 in which the autoimmune disease is multiple sclerosis.

28. The method of Claim 25 in which the autoimmune disease is rheumatoid arthritis.
·20

29. The method of Claim 25 in which the autoimmune disease is insulin-dependent diabetes mellitus.

30. A method of preventing transplantation rejection,
25 comprising administering a therapeutically effective amount of an interleukin 2-Bax chimeric protein to eliminate interleukin 2 receptor-positive cells.

31. A method of treating graft-versus-host disease,
30 comprising administering a therapeutically effective amount of an interleukin 2-Bax chimeric protein to eliminate IL2R-positive cells.

32. A method of treating post-ischemic immune response,
35 comprising administering a therapeutically effective amount of an interleukin 2-Bax chimeric protein to eliminate interleukin 2 receptor-positive cells.

33. A method of treating malignant or pre-malignant cells, comprising administering a therapeutically effective amount of a chimeric protein which comprises a cell-specific targeting moiety and an apoptosis-inducing moiety, and the 5 chimeric protein eliminates the malignant or pre-malignant cell.

34. The method of Claim 32 in which the cell-specific targeting moiety is gonadotrophin releasing hormone.
10
34. A method of detecting an IL2R-positive cell comprising contacting a cell mixture with IL2-Bax and selecting the cell bound by IL2-Bax.

15 35. A method of treating a hypersensitivy comprising administering a therapeutically effective amount of a chimeric protein which comprises an Fc portion of an IgE antibody and an apoptosis-inducing moiety.

20 36. A method of treating an infectious disease comprising administering a therapeutically effective amount of a chimeric protein which comprises a cell targeting moiety which binds an antigen of an infectious agent expressed by a cell and an apoptosis-inducing moiety.
25

30

35

FIG.1

```
1/1                                          31/11
atg gca gat cct act tca agt tct aca aag  aaa aca cag cta caa ctg gag cat tta ctg
Met ala asp pro thr ser ser ser thr lys  lys thr gln leu gln leu glu his leu leu

61/21                                        91/31
ctg gat tta cag atg att ttg aat gga att  tac aag aat ccc aaa ctc acc agg
leu asp leu gln met ile leu asn gly ile  tyr lys asn pro lys leu thr arg

121/41                                       151/51
atg ctc aca ttt aag ttt tac atg ccc aag  aag gcc aca gag ctg aaa cat ctt cag tgt
met leu thr phe lys phe tyr met pro lys  lys ala thr glu leu lys his leu gln cys

181/61                                       211/71
cta gaa gaa ctc aaa cct ctg gag gaa gga  gta cta aat tta gct caa agc aaa aac ttt
leu glu glu leu lys pro leu glu glu gly  val leu asn leu ala gln ser lys asn phe

241/81                                       271/91
cac tta aga ccc agg gac gac tta atc agc  aat gta ata gtt ctg gaa ctg aag gga
his leu arg pro arg asp asp leu ile ser  asn val ile val leu glu leu lys gly

301/101                                      331/111
tct gaa aca aca ttc atg tgt gaa tat gct  gat gag gaa tat gct aca gca acc att gta gaa ttt ctg
ser glu thr thr phe met cys glu tyr ala  asp glu glu tyr ala thr ala thr ile val glu phe leu

361/121                                      391/131
aac aga tgg att acc ttt tgt caa agc atc  atc tca aca atc ccc gag gga ggc gaa gct ttg
asn arg trp ile thr phe cys gln ser ile  ile ser thr ile pro glu gly gly glu ala leu

421/141                                      451/151
GAC GGG TCC GGG GAG CAG CCC AGA GGC GGG  ACC AGC TCT GAG CAG ATC ATC AAG
asp gly ser gly glu gln pro arg gly gly  thr ser ser glu gln ile ile lys

481/161                                      511/171
GGG GGG GAG  ACA GGG GCC CTT TTG CTT CAG GGT TTC ATC CAG GAT CGA GCA GGG CCA ATC GGG CGG GAG
gly gly glu  thr gly ala leu leu leu gln gly phe ile gln asp arg ala gly met gly gly glu
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FIG. 2A

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541/181
GCA CCC GAG CTG GCC CTG GAC CCG GTG·CCT
ala pro glu leu ala leu asp pro val pro

571/191
CAG GAT GCG TCC ACC AAG AAG CTG AGC GAG
gln asp ala ser thr lys lys leu ser glu

601/201
TGT CTC AAG CGC ATC GGG GAC GAA CTG GAC
cys leu lys arg ile gly asp glu leu asp

631/211
AGT AAC ATG GAG CTG CAG AGG ATG ATT GCC
ser asn met glu leu gln arg met ile ala

661/221
GCC GTG GAC ACA GAC TCC CCC CGA GAG GTC
ala val asp thr asp ser pro arg glu val

691/231
TTT TTC CGA GAG GTC GCA GCT GAC ATG TTT TCT
phe phe arg glu val ala ala asp met phe ser

721/241
GCC GTG GTG CGA GGG CGG GAG GTT GTC
ala val val arg gly arg glu val val

751/251
GAC GGC AAC TTC AAC TGG GGC CGG GTT GTC
asp gly asn phe asn trp gly arg val val

781/261
CTC AAG GCC CTG TGC ACC AAG GTG CCG GTG
leu lys ala leu cys thr lys val pro val

811/271
CCG GAA CTG ATC AGA ATC ATG GGC ACA TTG
pro glu leu ile arg ile met gly thr leu

841/281
GAC TTC CTC CGG GAG CGG CTG GGC CTG GGC
asp phe leu arg glu arg leu gly leu gly

871/291
ATC CAA GAC CAG GGT GGC TGG GAC GGC CTC
ile gln asp gln gly gly trp asp gly leu

901/301
TTC GGG ACG CCC ACG TGG CAG ACC GTG ACC
phe gly thr pro thr trp gln thr val thr

931/311
CTC TCC TAC TTT GTG GCG ACC ATC TTT GTG
leu ser tyr phe val ala thr ile phe val

961/321
ACC GCC TCG CTC ACC ATC TGG AAG AAG ATG GGC TGA
thr ala ser leu thr ile trp lys lys met gly QPA

991/331
```

FIG.2B

FIG.3

FIG.4

FIG.5A

FIG.5B

DEXAMETASONE
M1=18.96
M2=3.28

FIG.6B

CONTROL
M1=6.3
M2=1.56

FIG.6A

IL2–BAX. 10μg

M1=47.04
M2=12.96

M2

M1

FIG.6D

IL2–BAX. 1μg

M1=29.86
M2=5.06

M2

M1

FIG.6C

CONTROL
UL=2.35
UR=1.56

10^4

10^3

10^2

10^1

10^0

0   200   400   600   800   1000

FIG.7A

IL-Bax. 1μg
UL=4.98
UR=5.56

10^4

10^3

10^2

10^1

10^0

0   200   400   600   800   1000

FIG.7B

IL2-Bax. 10µg
M1=17.04
M2=22.72

FIG.7C

FIG.8B

FIG.8A

4μg/ml IL2-BAX
M1=15.11%

FIG.8D

2μg/ml IL2-BAX
M1=8.23%

FIG.8C

FIG.8E

FIG.9A

FIG.9B

FIG.9C